

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 0704-0188

AD-A267 111



is estimated to average 1 hour per response, including the time for reviewing instructions, searching existing data sources, gathering and reviewing the collection of information. Send comments regarding this burden estimate or any other aspect of this collection of information, including suggestions for reducing this burden, to Washington Headquarters Services, Directorate for Information Operations and Reports, 1215 Jefferson Avenue, Alexandria, VA 22304-6146, and to the Office of Management and Budget, Paperwork Reduction Project (0704-0188), Washington, DC 20503.

REPORT DATE

3. REPORT TYPE AND DATES COVERED

Affinity purification and subcellular localization of kinesin in human neutrophils

5. FUNDING NUMBERS

6. AUTHOR(S)

Stephen W. Rothwell, Carolyn C. Deal, Jay Pinto and Daniel G. Wright

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Walter Reed Army Institute of Research
Washington, D.C. 20307-51008. PERFORMING ORGANIZATION
REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Development Command
Ft. Detrick, Frederick, MD 21702-501210. SPONSORING / MONITORING
AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

DTIC
ELECTE
JUL 23 1993
S B D

12a. DISTRIBUTION / AVAILABILITY STATEMENT

APPROVED FOR PUBLIC RELEASE:
DISTRIBUTION UNLIMITED

12b. DISTRIBUTION CODE

Not for circulation
This document is a product
of the DTIC and will be in black and
white.

13. ABSTRACT (Maximum 200 words)

Studies of granule-microtubule interactions in human neutrophils have suggested that mechanochemical ATPases such as kinesin or dynein may play a role in granule mobilization during neutrophil activation by inflammatory signals. In this study we show that proteins extracted from the surface of neutrophil granules, found previously to contain microtubule-dependent ATPase activity, caused microtubules polymerized from phosphocellulose-purified rat brain tubulin to move across glass slides. Antibodies were generated against peptides based on two regions of the amino acid sequence of *Drosophila* kinesin: the ATPase active site (amino acids 86-99) in the head of the kinesin heavy chain and the tail of the heavy chain (residues 913-933). These antibodies were found to recognize kinesin in rat brain extracts as well as kinesin-like polypeptides in extracts of human neutrophils. Furthermore, when used in immunoaffinity chromatography, these antibodies permitted the isolation of a protein from neutrophil granule extracts that was recognized by *Drosophila* kinesin antibodies. Subcellular localization by immunofluorescence microscopy showed this protein to be associated principally with the cytoplasmic granules of neutrophils.

14. SUBJECT TERMS

neutrophils, kinesin, microtubules, peptides, granules

15. NUMBER OF PAGES

16. PRICE CODE

17. SECURITY CLASSIFICATION
OF REPORT18. SECURITY CLASSIFICATION
OF THIS PAGE19. SECURITY CLASSIFICATION
OF ABSTRACT

20. LIMITATION OF ABSTRACT

Affinity purification and subcellular localization of kinesin in human neutrophils

Stephen W. Rothwell, Carolyn C. Deal,* Jay Pinto, and Daniel G. Wright

Departments of Hematology and *Bacterial Diseases, Walter Reed Army Institute of Research, Washington, DC

Abstract: Studies of granule-microtubule interactions in human neutrophils have suggested that mechanochemical ATPases such as kinesin or dynein may play a role in granule mobilization during neutrophil activation by inflammatory signals. In this study we show that proteins extracted from the surface of neutrophil granules, found previously to contain microtubule-dependent ATPase activity, caused microtubules polymerized from phosphocellulose-purified rat brain tubulin to move across glass slides. Antibodies were generated against peptides based on two regions of the amino acid sequence of *Drosophila* kinesin: the ATPase active site (amino acids 86-99) in the head of the kinesin heavy chain and the tail of the heavy chain (residues 913-933). These antibodies were found to recognize kinesin in rat brain extracts as well as kinesin-like polypeptides in extracts of human neutrophils. Furthermore, when used in immunoaffinity chromatography, these antibodies permitted the isolation of a protein from neutrophil granule extracts that was recognized by *Drosophila* kinesin antibodies. Subcellular localization by immunofluorescence microscopy showed this protein to be associated principally with the cytoplasmic granules of neutrophils. *J. Leukoc. Biol.* 53: 372-380; 1993.

Key Words: neutrophils • kinesin • microtubules • peptides • granules

INTRODUCTION

The directed intracellular movement of secretory vesicles and granules in most cells is now believed to be dependent on discrete translocation systems composed of biological polymers such as microtubules or actin filaments in conjugation with mechanochemical enzyme complexes that both link the organelles to the polymers and propel them along this underlying framework [1, 2].

Characterization of the molecular motors that provide the motive force for the translocation of secretory granules and other intracellular organelles has proceeded rapidly. Work by Adams and Pollard [3] and Kachar and Reese [4] has demonstrated the ability of myosin I to move organelles along actin filaments. In a separate organelle translocation system, the enzyme kinesin has been shown to mediate organelle movement along microtubules toward the plus end of microtubule polymers [5], and cytoplasmic dynein has been shown to mediate minus end-directed movement of organelles along microtubules [6].

Human neutrophils contain a heterogeneous population of cytoplasmic granules that are mobilized for extracellular secretion as well as fusion with endosomal vesicles and phagosomes in response to external stimuli. Our previous investigations have shown that neutrophil granules interact with microtubules in an ATP-dependent manner and that salt extracts of granules display microtubule-dependent

Pase activities characteristic of the vesicle-motor enzymes, dynein and kinesin [7].

In this study we report that proteins recoverable by salt extraction from intact neutrophil granules are capable of causing microtubule gliding at a slow but uniform rate of movement. To investigate the composition of granule extracts, we produced polyclonal antibodies generated against two distinct regions of the amino acid sequence of *Drosophila* kinesin. These regions included the putative ATPase active site in the head of the kinesin heavy chain and the organelle binding site near the tail of the heavy chain [8]. These antibodies bound specifically to rabbit and rat brain kinesin as well as to a kinesin-like protein in salt extracts of human neutrophil granules and were used for affinity purification of the neutrophil kinesin-like polypeptide. Furthermore, when these antibodies were used to label resting neutrophils for immunofluorescence microscopy, antibody labeling was found to be localized to neutrophil granules as well as to the microtubule network.

METHODS

Synthesis of peptides

Using the *Drosophila* kinesin heavy chain sequence determined by Yang et al. [8], two peptide sequences were selected for synthesis that correspond to the active site of the ATPase in the head region (amino acids 86-99 = head) and a possible organelle binding site in the tail (amino acids 913-933 = tail). The peptides were synthesized on an Applied Biosystems 530A (Foster City, CA) by the method of Merrifield [9] using tert. Butyloxycarbonyl protected amino acids and purified by reverse-phase high-performance liquid chromatography. The identity of the peptides was confirmed by amino acid analysis.

Antibody production

Polyclonal antibodies were produced in male New Zealand rabbits. The peptides were conjugated to keyhole limpet hemocyanin with glutaraldehyde and mixed with phosphate-buffered saline (PBS) and Freund's incomplete adjuvant for subcutaneous injection. Antibodies were isolated from the rabbit serum using DEAE Affi-gel Blue (Bio-Rad Laboratories, Richmond, CA) columns equilibrated in 20 mM Tris-HCl containing 28 mM NaCl. Specific immunoglobulins were affinity purified using an affinity matrix prepared from Bio-

Abbreviations: BSA, bovine serum albumin; IgG, immunoglobulin G; PBS, phosphate-buffered saline; PC, phosphocellulose; PEM, PIPES, EDTA, MgCl₂ buffer; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis.

Reprint requests: Stephen W. Rothwell, Division of Medicine, Walter Reed Army Institute of Research, Washington, DC 20307-5100.

Received January 2, 1992; accepted January 22, 1993.



Rad Affi-gel Hz and bovine serum albumin (BSA)-peptide conjugates. Antibodies were bound and eluted using the Pierce Gentle Ag/Ab buffer system (Pierce, Rockford, IL).

Protein preparation

Neutrophils were isolated from the venous blood of healthy volunteers by centrifugation through Ficoll-Paque (Pharmacia, Piscataway, NJ), followed by dextran sedimentation and removal of residual red blood cells by hypotonic lysis as described previously [10]. The cells were suspended in 10 mM PIPES, pH 6.9, 100 mM KCl, 3 mM NaCl, 1 mM phenylmethyl sulfonylfluoride (PMSF), 0.1 mM leupeptin, and 0.1 mg/ml aprotinin [11] containing 1 mM ATP and disrupted by sonication or nitrogen cavitation. Unbroken cells and nuclei were removed from the cell sample by centrifugation at 400g for 10 min. The cell extract was further fractionated into cytosol and granules either by centrifugation (14,000g for 20 min) or by passing the postnuclear supernatant over a Sepharose 6B column to separate intact granules from cytoplasmic proteins. The granules were treated with 1 M KCl to extract granule-associated proteins and exchanged into either 100 mM PIPES, pH 6.9, 1 mM EGTA, 1 mM MgCl₂ with proteolytic inhibitors (PEM buffer) or Pierce Gentle Binding Buffer (Rockford, IL) by gel filtration. Protein samples from whole cell extracts, cytosol, or granule extracts were passed over affinity gels prepared using the affinity-purified antibodies coupled to Affi-gel Hz (Bio-Rad Laboratories) and eluted with Pierce Gentle Elution Buffer to isolate immunoreactive polypeptides.

Kinesin was isolated from rat brains according to the method of Johnson et al. [12]. Brains were homogenized in 100 mM PIPES, pH 6.94, 2.5 mM MgCl₂, 5 mM EGTA, 0.5 mM EDTA, 1.0 mM β -mercaptoethanol, 5% glycerol, and proteolytic inhibitors. The extract was enriched in kinesin by ion exchange chromatography using Whatman P-11 phosphocellulose (PC) and the eluted proteins concentrated by ammonium sulfate. The kinesin was further purified by a modification of the microtubule binding procedure using tripolyphosphate to enhance binding of kinesin to the microtubules and then concentrated with a second P-11 treatment.

Bovine kinesin was also obtained as a purified protein from D. Murphy (Johns Hopkins School of Medicine, Baltimore, MD).

Microtubule motility assays

Tubulin for the microtubule gliding experiments was isolated from bovine brain tissue [13] or chicken erythrocytes [14] in 0.1 M PIPES buffer containing 1 mM MgCl₂, 2 mM EGTA, 1 mM GTP, and 4 M glycerol. The tubulin was purified free of microtubule-associated proteins by ion exchange chromatography using Whatman P-11 PC [15] and processed through a cycle of microtubule assembly and disassembly prior to use in order to remove any inactive subunits. Microtubule assembly buffer was 0.1 M Na-PIPES at pH 6.94 containing 1 mM MgCl₂, 1 mM GTP, and supplemented with 5% glycerol. Microtubules were then polymerized from this protein preparation and stabilized with 10 μ M taxol. Granule extracts (25 μ l), prepared as above in PEM buffer, were allowed to adhere for 30 s to glass coverslips and washed with PEM buffer, and then microtubules (6 μ l, 0.1–0.2 mg/ml) and ATP (1 μ l, 100 mM stock) were added. Microtubule movement was viewed in an Orthoplan microscope (E. Leitz, Rockleigh, NJ) equipped with a 100 \times /1.32 numerical aperture NPL/Fluotar ICT objective and

a 12-V, 100-W tungsten halogen lamp. Images were projected to a Hitachi video camera (Hitachi-Denshi, Tokyo, Japan) and then further processed by an image processor (model DS-580; Quantex, Sunnyvale, CA). The processed images were recorded with a JVC CR 6060U 3/4-inch videocassette recorder.

SDS-PAGE and immunoblotting

Conditions for sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were as previously described by Murphy and Wallis [14]. Proteins were fractionated on 10% acrylamide/DATD (*N,N'*-diallyltartardiamide) gels, pH 8.8, and transferred to nitrocellulose paper [16]. The papers were incubated in solutions containing antibodies specific for the different head or tail peptide or *Drosophila* kinesin (gift of Dr. J. Scholey, University of California, Davis). Antibody-antigen complexes were visualized after washing with Tris-buffered saline containing 0.05% Tween-20 by addition of 100 mM Tris, pH 9.5, containing 100 mM NaCl, 5 mM MgCl₂, and 6.6 μ l/ml of a stock solution of 50 mg/ml nitroblue tetrazolium in 50% dimethyl formamide and 3.3 μ l/ml of a stock solution of 50 mg/ml 5-bromo-4-chloro-3-indolylphosphate in 100% dimethyl formamide.

Immunofluorescence microscopy

Neutrophils in Hanks' balanced salt solution without calcium or magnesium (50 μ l at concentrations of 1.4 to 2 million cells per ml) were allowed to spread at 37°C for 25 min on square glass coverslips. The coverslips were then incubated for 30 s at 37°C in two changes of stabilization buffer (100 mM PIPES, 1 mM EGTA, 5 mM MgCl₂, 4% polyethylene glycol 6000, 2.5 mM GTP) [17]. Cells to be labeled with kinesin antibodies were extracted in prewarmed stabilization buffer containing 0.5% Triton X-100 for 2 min. Extracted cells were fixed in a solution of 3% formaldehyde for 8 min and then washed three times in PBS prior to the application of the primary antibody. Affinity-purified antikinesin antibodies prepared against peptides synthesized using the sequence of the tail or head of *Drosophila* kinesin was used at a 1:5 dilution. For microtubule labeling, cells were extracted in prewarmed stabilization buffer containing 0.5% Triton X-100 for 8 min. When cells were double labeled the cells were treated as for kinesin labeling. The decreased extraction time with detergent limited the loss of kinesin but slightly degraded the image of the microtubule staining. The extracted cells were fixed in a solution of 2% formaldehyde and 0.1% glutaraldehyde in PBS for 10 min and immediately placed in sodium borohydride (1 mg/ml) for 1 min. Cells were then dehydrated in -20°C methanol for 6 min, followed by 1 min in -20°C acetone. The coverslips were washed three times in PBS prior to the application of primary antibody solution. For single-antibody staining of microtubules a rabbit antitubulin antibody specific for tyrosinated tubulin (gift of Dr. J. Bulinski, Columbia University, New York) was used at a 1:250 dilution. For double labeling of kinesin and microtubules, a rat antibody specific for tyrosinated tubulin (Accurate Chemicals, Westbury, NY) was used. Each coverslip was incubated with 50 μ l of diluted antibody for 1 h in a humid chamber. Coverslips were then washed three times for 5 min each in PBS prior to the addition of the secondary fluorescent antibody. Then 50 μ l of antirabbit immunoglobulin G (IgG) fluorescein F(ab')₂ antibody (Boehringer Mannheim GmbH) or antirat IgG rhodamine antibody (Pierce) at a concentration of 5 μ g/ml was added to each coverslip. Coverslips were then in-

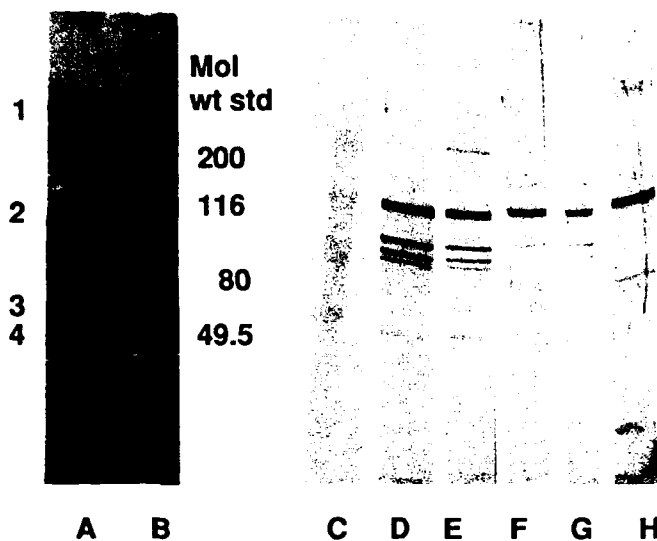


Fig. 1. Antikinesin antibodies generated against synthetic peptides derived from amino terminal (head) and carboxyl terminal (tail) sequences of *Drosophila* kinesin and against intact *Drosophila* kinesin recognize kinesin in kinesin-enriched rat brain samples. Lane A, silver-stained rat brain kinesin preparation. [The major protein bands in the preparation are tentatively identified as dynein (1), kinesin (2), bovine serum albumin (3), and tubulin (4). BSA and tubulin were added as carrier molecules.] Lane B, silver-stained molecular weight markers; lane C, molecular weight markers, blotted to nitrocellulose; lanes D and E, kinesin preparation probed with tail kinesin antibodies raised in two different rabbits; lanes F and G, kinesin preparation probed with head kinesin antibodies raised in two different rabbits; lane H, kinesin preparation probed with antibody against intact *Drosophila* kinesin.

cubated for 1 h at 37°C in humid chambers. After washing three times in PBS, coverslips were mounted on precleaned slides (Esco Scientific Company, Portsmouth, NH) with Vectashield (Vector, Burlingame, CA). The edges of the coverslips were sealed and slides refrigerated at 4°C until ready to be viewed in a Leitz Orthoplan microscope. Pictures were taken using Kodak Ektapress 400 film shot at 800 ASA.

For immunofluorescence staining of neutrophil granules, the cells were disrupted by nitrogen cavitation and the cavitated samples layered onto 30–60% continuous sucrose gradients made using a Gradient Master gradient maker (BioComp, New Brunswick, Canada). The gradients were centrifuged at 100,000g for 7 h and fractions containing plasma membrane vesicles and cytoplasmic granules were collected. Drops (50 μ l) of the granules or vesicles were placed on cleaned glass slides, fixed in a solution of 2% formaldehyde and 0.1% glutaraldehyde for 10 min, and immediately placed in sodium borohydride (1 mg/ml) for 1 min. The slides then were incubated with antibodies against either kinesin or tubulin and washed with PBS before incubation with the fluorescein-labeled antibodies as described earlier.

RESULTS

Salt extracts of human neutrophil granules cause microtubule gliding

The ability of protein preparations and cytoplasmic extracts to move microtubules across glass coverslips has been used as an indicator of the presence of microtubule motor enzymes such as kinesin or dynein [18]. In initial studies, neutrophil cytosol, either unmodified or processed through sequential steps of incubation with microtubules and

nucleotide extraction to enrich for ATP-sensitive proteins, failed to cause microtubule gliding. However, when salt extracts of neutrophil granules were used in the microtubule gliding assay, they were found to induce a slow but consistent rate of movement (0.05 μ m/s) that proceeded without detectable interruptions. This movement was reproduced using microtubules from either bovine brain tissue or chicken erythrocytes, indicating ability of the neutrophil-derived motor molecules to function with microtubules formed from tubulin of different species.

Antikinesin peptide antibodies recognize neuronal kinesin

To further define components of the salt-extractable granule protein preparations, we produced polyclonal antibodies generated against specific sequences of the *Drosophila* kinesin molecule. These sequences were selected from regions near the ATPase active site (amino acids 86–99) in the kinesin heavy chain head and near the tail of the heavy chain (residues 913–933). These areas of the molecule were assumed to be essential to the normal functions of the molecule and, therefore, likely to be conserved across interspecies boundaries.

As shown in Figure 1, the antibodies produced against both the tail and head sequences recognized kinesin in kinesin-enriched samples prepared from rat brain extracts. Antibodies against the tail sequences (Fig. 1, lanes D and E) in addition recognized three lower-molecular-weight bands that were not detected by the antibodies against the head sequences (Fig. 1, lanes F and G). To confirm the specificity of the antibodies, the binding pattern of the peptide antibodies was compared to the bands recognized by an antibody produced against intact *Drosophila* kinesin (Fig. 1, lane H). All five antibody preparations were able to detect the kinesin heavy chain.

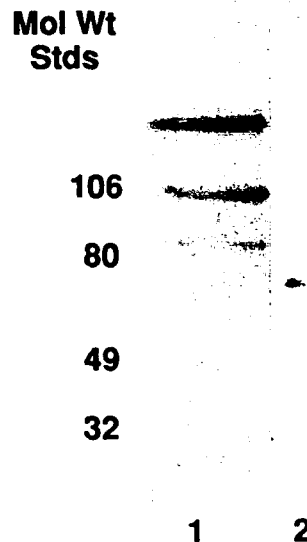


Fig. 2. Antibodies raised against different sequences from the head and tail regions of *Drosophila* kinesin heavy chain recognize different polypeptide fragments of purified bovine brain kinesin. Protein degradation was induced in rat brain kinesin by freeze-thaw cycles and the polypeptides were probed by immunoblotting with the tail and head kinesin antibodies. Lane 1, kinesin fragments probed with kinesin tail antibody; lane 2, kinesin fragments probed with kinesin head antibody. The positions of molecular mass markers are indicated beside the blot.

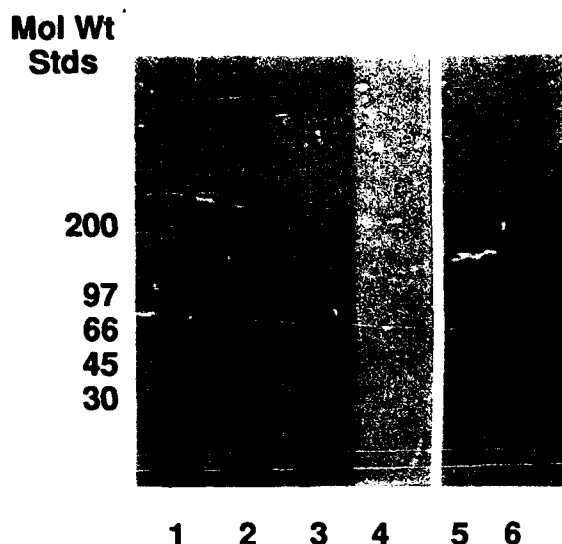


Fig. 3. Identical proteins are recognized in human neutrophil whole cell extracts and granule extracts by antibodies raised against both peptide sequences as well as antibodies against intact kinesin. Immunoblots against whole neutrophil cell samples were performed with the following reagents: lane 1, *Drosophila* kinesin antibody; lane 2, tail peptide antibody; lane 3, head peptide antibody; lane 4, blocked tail antibody (before incubation with the nitrocellulose strip, tail antibody was precubated with BSA-peptide conjugate for 1 h). Immunoblots against proteins removed from the exterior of neutrophil granules by KCl extraction were performed with the following reagents: lane 5, tail peptide antibody; lane 6, head peptide antibody.

Although both the tail and head kinesin antibodies recognized the same intact molecule, it was clear that the antibodies bind to different regions of the polypeptide. Bovine brain kinesin (independently purified by the laboratory of D. Murphy, Johns Hopkins School of Medicine) was also recognized by the tail and head kinesin antibodies. When this form of kinesin was subjected to several cycles of freeze-thawing, partial degradation occurred and produced fragments of different sizes. As shown in **Figure 2**, the head antibody recognized principally a low-molecular-mass fragment. On the other hand, the tail antibody recognized two larger fragments, in addition to a polypeptide close in size to the intact molecule. Analogous results were obtained when peptide antibodies were blotted to kinesin preparations that had been partially digested with chymotrypsin (data not shown).

Antikinesin peptide antibodies recognize kinesin-like proteins in human neutrophils

The antikinesin antibodies were used to probe for the presence of kinesin in neutrophil samples. Immunoblots of whole neutrophil extracts showed that kinesin antibodies against the head and tail kinesin sequences as well as *Drosophila* kinesin reacted primarily with a protein with an apparent molecule mass of 90–100 kD and several bands of low molecular mass (**Fig. 3**, lanes 1–3). Blocking of the binding of the tail antibody to the protein bands was demonstrated by incubation of the tail kinesin antibody with its corresponding bovine serum albumin (BSA)-peptide conjugate (**Fig. 3**, lane 4).

Since the ATPase activity reported in our previous studies was detected in protein samples recovered from neutrophil granules following KCl extraction, samples prepared in this manner were probed with the head and tail kinesin antibodies. As in our experiments with whole neutrophil extracts, the antibodies again detected protein bands in the 90–100 kD range and at < 30 kD (**Fig. 3**, lanes 5 and 6) but not at the

position of 115 kD, where intact neural kinesin had been seen. The protein band in neutrophil samples at 100 kD was observed repeatedly using both sets of kinesin antibodies despite the inclusion of high concentrations of proteolytic inhibitors in the buffers at all stages of sample preparation.

Purification of a kinesin-like protein from neutrophil extracts by affinity chromatography

Isolation of the immunoreactive polypeptide from both whole cell extracts and granule extracts was achieved by affinity chromatography. As shown in **Figure 4**, a polypeptide of the same apparent molecular mass as observed previously in immunoblots of whole neutrophil samples and granule extracts was also isolated by affinity chromatography from whole cell extracts of human neutrophils using both the head and tail antibodies. However, despite the inclusion of multiple proteolytic inhibitors, including diisopropyl fluorophosphate, PMSF, aprotinin, chymostatin, and leupeptin, the apparent molecular mass of the protein recovered from the granule extracts (≈ 95 kD) was consistently lower than that of the polypeptides recognized by immunoblotting brain (115 kD). If the columns were isolating different proteolytic fragments of similar size, one would not expect the antibodies against the head peptide to recognize the protein isolated by the tail kinesin antibody column or the tail peptide antibody to recognize the protein recovered from the head antibody column. However, the polypeptides collected from affinity columns constructed using either the head or tail kinesin antibodies were detected by both the head antibody (**Fig. 4A**, lanes 5 and 6) and the tail antibody (**Fig. 4B**, lanes 4 and 5).

Subcellular localization of neutrophil kinesin

Immunofluorescence microscopy was performed using the kinesin peptide antibodies to determine the subcellular location of kinesin in human neutrophils. As shown in **Figure 5C–F**, immunostaining with the kinesin antibodies gave a punctate pattern throughout the region of the cytoplasm. Examination of the regions that were stained by the antikinesin antibodies suggested that the antibody binding was localized to neutrophil granules (**Fig. 5B and C**). This hypothesis was strengthened by the observation that extraction procedures that completely removed the cell membranes also eliminated kinesin-specific staining. When granules were separated from cytosol and low-density plasma membrane-derived vesicles, we saw that the antikinesin antibodies were able to label the granules but not plasma membrane vesicles (**Fig. 6**). Antitubulin antibody, run as a control, was unable to label either granules or vesicles. This apparent localization of kinesin to the cytoplasmic granules of neutrophils is in agreement with our previous demonstration of a granule-associated kinesin-like molecule and microtubule-dependent ATPase activity in human neutrophils [7]. This finding is also consistent with the observations of Pfister et al. [19], who found kinesin to be localized to the surface of vesicles isolated from brain and epithelial cells.

A second question concerning the localization of kinesin was whether kinesin was also associated with the microtubules. Comparison of cells labeled only with antikinesin antibody to cells labeled only with antitubulin antibodies did not immediately indicate whether the kinesin could also be localized to microtubules (**Fig. 5**). However, cells treated for optimal labeling of microtubules were subjected to extensive detergent extraction, which removed the granule-associated kinesin staining and, possibly, any kinesin associated with

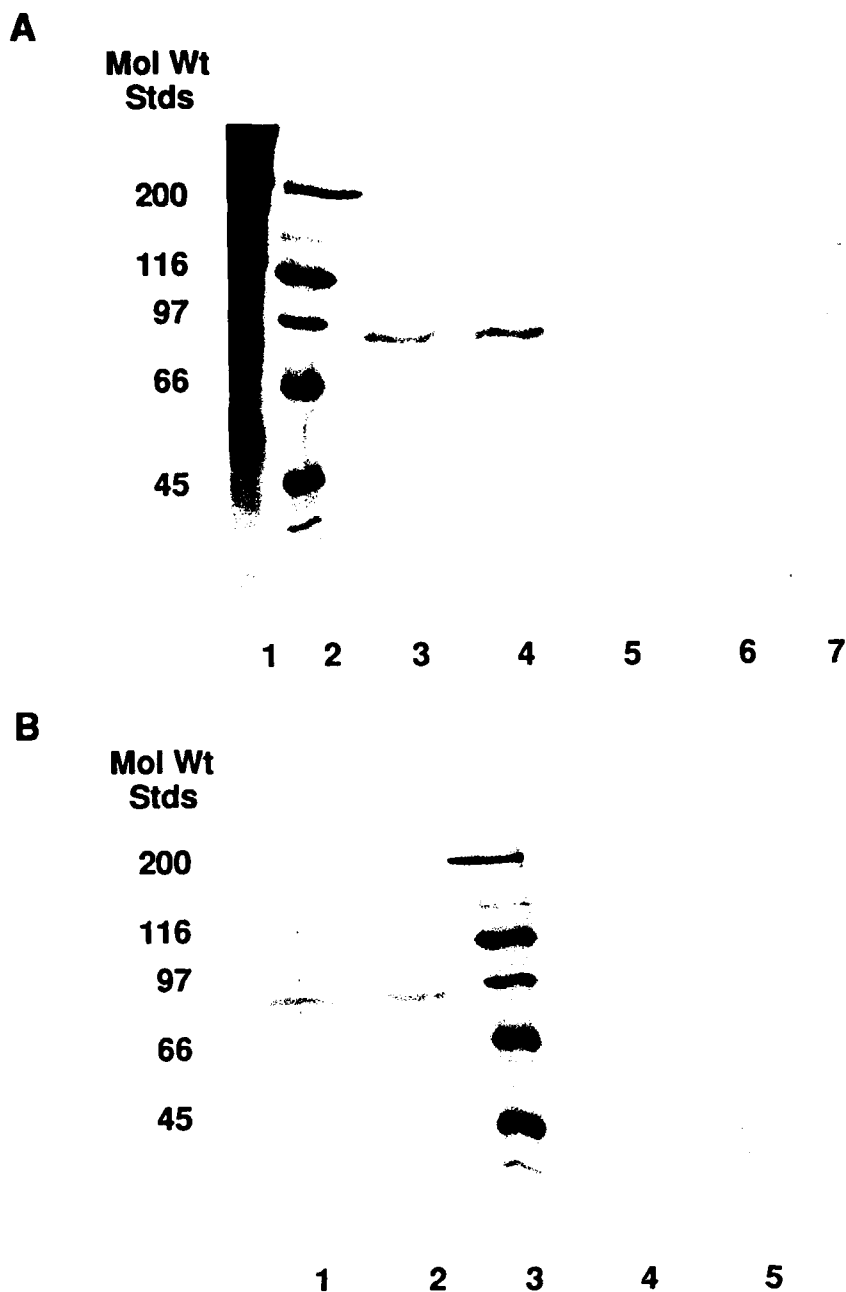


Fig. 4. Affinity chromatography isolates a kinesin-like protein from human neutrophils. Whole neutrophil cell samples were incubated with affinity matrices coupled to antitail and antihelix kinesin antibodies. Proteins recovered from the two columns were analyzed by SDS-PAGE and immunoblotting. Panels A and B represent two separate affinity purification experiments. (A) Lane 1, prestained molecular mass markers; lane 2, silver-stained molecular standards (apparent molecular masses refer to the silver-stained standards); lane 3, proteins eluted from head antibody affinity column (silver-stained gel); lane 4, proteins eluted from tail antibody affinity column (gel); lanes 5 and 6, immunoblots against the proteins shown in lanes 1 and 2 using antibodies against tail peptides; lane 7, prestained standards (equivalent to lane 1). (B) Lane 1, proteins eluted from head antibody affinity column (silver-stained gel); lane 2, proteins eluted from tail antibody affinity column (gel); lane 3, silver-stained molecular standards; lanes 4 and 5, immunoblots against the proteins shown in lanes 1 and 2 using antibody against the head peptide.

microtubules. On the other hand, cells treated for kinesin staining were exposed to detergent only briefly, a technique that preserved kinesin staining but did not yield clear microtubule staining. Therefore, cells were double labeled using a procedure that was a compromise between the two extreme conditions. With this approach we detected a filamentous staining pattern in cells incubated with the antikinesin antibodies that matched the path of microtubules labeled by antitubulin antibodies (Fig. 7).

DISCUSSION

In this study we produced specific peptide antibodies directed against known regions of the *Drosophila* kinesin molecule that may be critical to the normal function of this protein. Using these reagents, we successfully identified kinesin-like polypeptides in samples prepared from whole human neutrophils and in extracts of neutrophil granules.

Isolation of immunologically related polypeptides from both whole cell preparations and granule extracts was then achieved by affinity chromatography. We also obtained evidence by immunofluorescence microscopy for binding of the kinesin-like protein to neutrophil granules and microtubules in situ. The immunofluorescence pattern of punctate staining is similar to that observed by Lin and Collins [20] for cytoplasmic dynein in mouse 3T3 cells and Pfister et al. [19] for kinesin in PtK₁ cells and suggests that, in neutrophils, a large proportion of the kinesin is associated with the granules. This staining pattern was further substantiated by the immunofluorescent staining of isolated neutrophil granules. It is interesting to note that, although most of the granules were brightly stained, in any field of granules there were always some granules that were not labeled by the antibodies. Plasma membrane-derived vesicles displayed no detectable fluorescent labeling with either of the kinesin peptide antibodies. Attempts to quantify the proportion of granular versus cytoplasmic kinesin were unsuccessful because of the

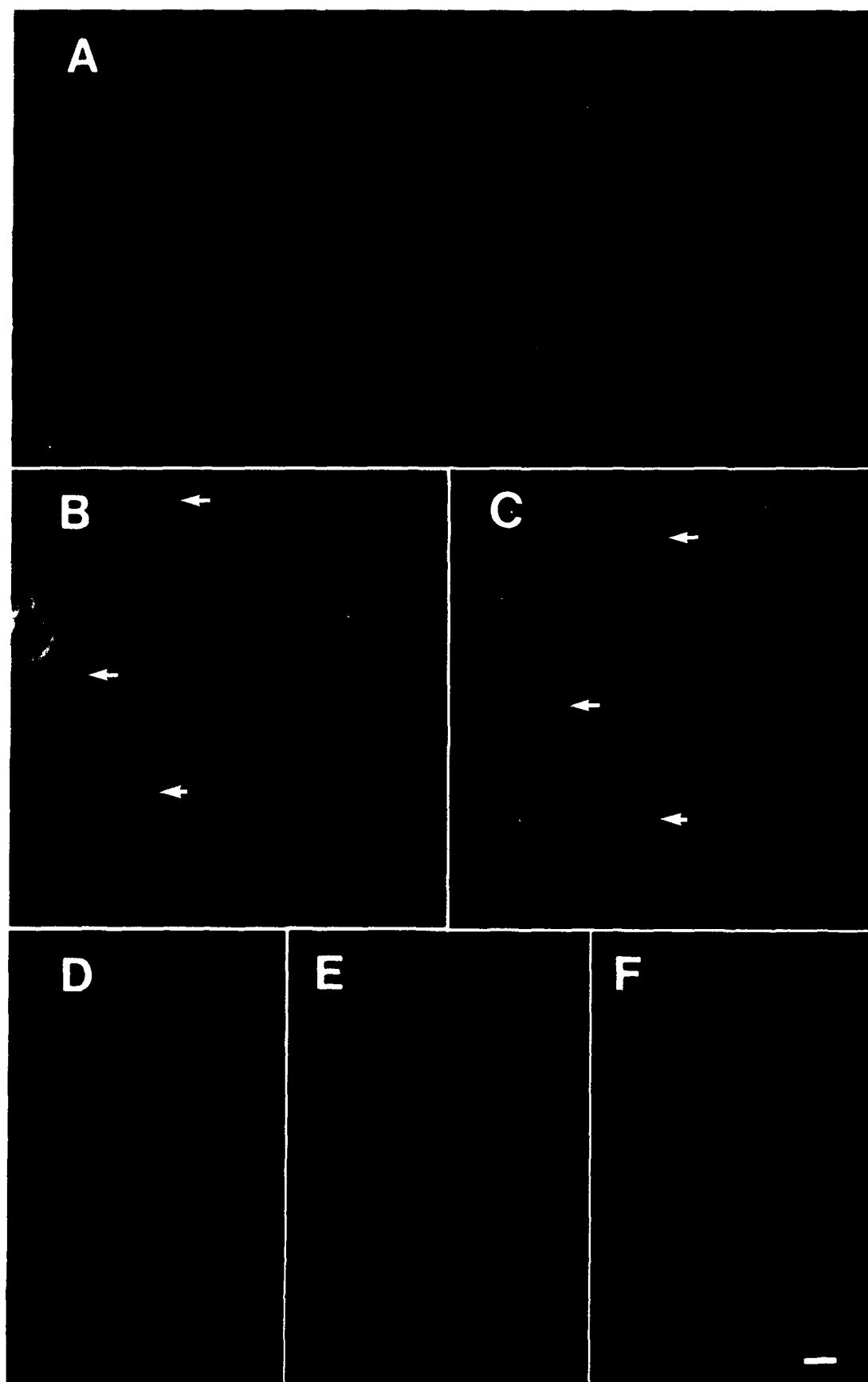


Fig. 5. Immunofluorescence microscopy of human neutrophils using antibodies against kinesin and tubulin. After fixation, cells were incubated with antitubulin antibody (A), affinity-purified antikinesin head antibodies (C and D), or affinity purified antikinesin tail antibodies (E and F). (B) Corresponding differential interference contrast image of (C). Arrows indicate clusters of granules in the two micrographs labeled by the antikinesin antibodies. Bar, 0.6 μ m.

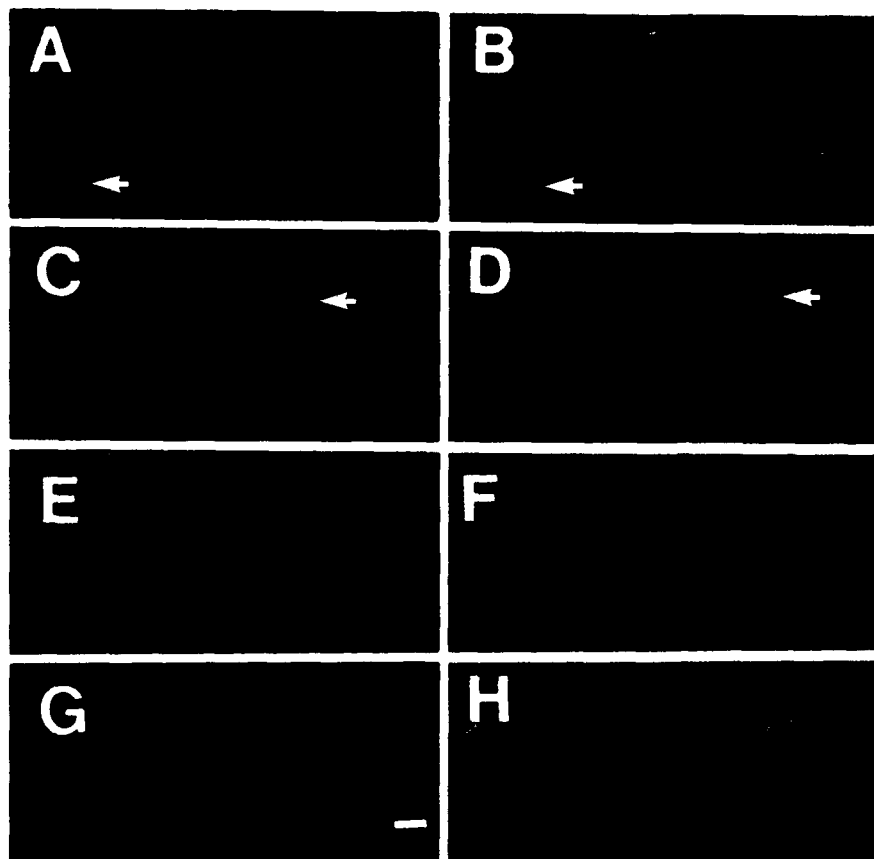


Fig. 6. Immunofluorescence microscopy of vesicles and granules isolated from human neutrophils. Neutrophil granules and low-density vesicles (plasma membrane) were recovered from disrupted cells and isolated on sucrose gradients. The samples and antibody combination are: (A) (B = corresponding DIC image), granules incubated with antikinesin head antibodies; C (D = corresponding DIC image), granules incubated with antikinesin tail antibodies; E (F = corresponding DIC image), vesicles incubated with antikinesin tail antibodies; G (H = corresponding DIC image), granules incubated with antitubulin antibodies. Arrows indicate corresponding granules in matched pairs of micrographs. Bar, 0.3 μ m.

almost undetectable levels of kinesin observed in cytosolic samples by immunoblotting.

The localization of kinesin principally to the cytoplasmic granules also agrees with the results of our previous enzymatic studies of microtubule-granule interactions in human neutrophils [18]. In earlier attempts to purify kinesin from neutrophils using biochemical techniques, we failed to detect microtubule-dependent ATPase activity in cytosol preparations. However, neutrophil granules displayed measurable microtubule-dependent ATPase activity that was extractable with 1 M KCl. In addition, as we report in this study, these salt extracts were found to propel microtubules across a glass surface. The levels of ATPase activity recovered from granule extracts and the rate of microtubule gliding induced by these extracts were low compared to these activities reported in other studies of microtubule-based molecular motors. This may reflect insufficiencies in the *in vitro* system, such as absence of accessory proteins or post-translational modifications of the neutrophil protein. Indeed, the kinesin-like protein purified from neutrophils was found to be smaller than the 115 kD reported for kinesin isolated from brain tissue. In addition, in our previous studies of neutrophil granule-associated ATPases we observed that azido-ATP could be used to photolabel a 115-kD polypeptide in the granule extracts [7]. This discrepancy in apparent sizes could be accounted for by either partial proteolytic cleavage or the presence in neutrophils, as in other eukaryotic cells [21], of multiple forms of kinesin with different physical properties and different cellular functions. If a cleavage event is occurring, there is also the question of whether it is induced during purification of the molecule or whether this modification has physiologic significance. Experiments in which the cells were isolated and plunged

directly into boiling denaturing and reducing buffer still yielded samples containing the lower-molecular-weight form of kinesin. This suggests that the smaller molecule exists in the living cell but does not by itself distinguish between the possibility of a posttranslational modification of a conventional kinesin heavy chain and the existence of a smaller kinesin transcript product. In blots of neutrophil samples, the antikinesin antibodies often detected low-molecular-weight proteins and binding to these protein bands could be eliminated if the antibodies were incubated with the peptides before exposure to the nitrocellulose membranes. This would indicate that a cleavage event generates the low-molecular-weight polypeptides.

The peptide antibodies generated in this study were designed to bind to specific sequences located at either end of the kinesin heavy chain molecule. Using the sequence data for the *Drosophila* kinesin molecule as a guide, the removal of the approximately 140 amino acid residues required to reduce the molecular mass from the range of 115 kD to 100 kD may be expected to delete the sections of the polypeptide containing the antibody sequences, since the head sequence was only 83 amino acid residues from the amino terminus and the tail sequence ended 42 residues from the carboxyl terminus. However, the smaller form of kinesin was recognized by both sets of peptides of kinesin antibodies.

In secretory cells, movement of organelles or granules is only one component of an integrated series of cellular events that are triggered by external stimuli and result in exocytosis responses. Studies of vesicle and granule movement systems have suggested a number of control mechanisms that involve the formation and dissolution of protein complexes between ATPases and accessory proteins. For example, a report by Schroer and Sheetz [22] indicates that dynein-binding pro-

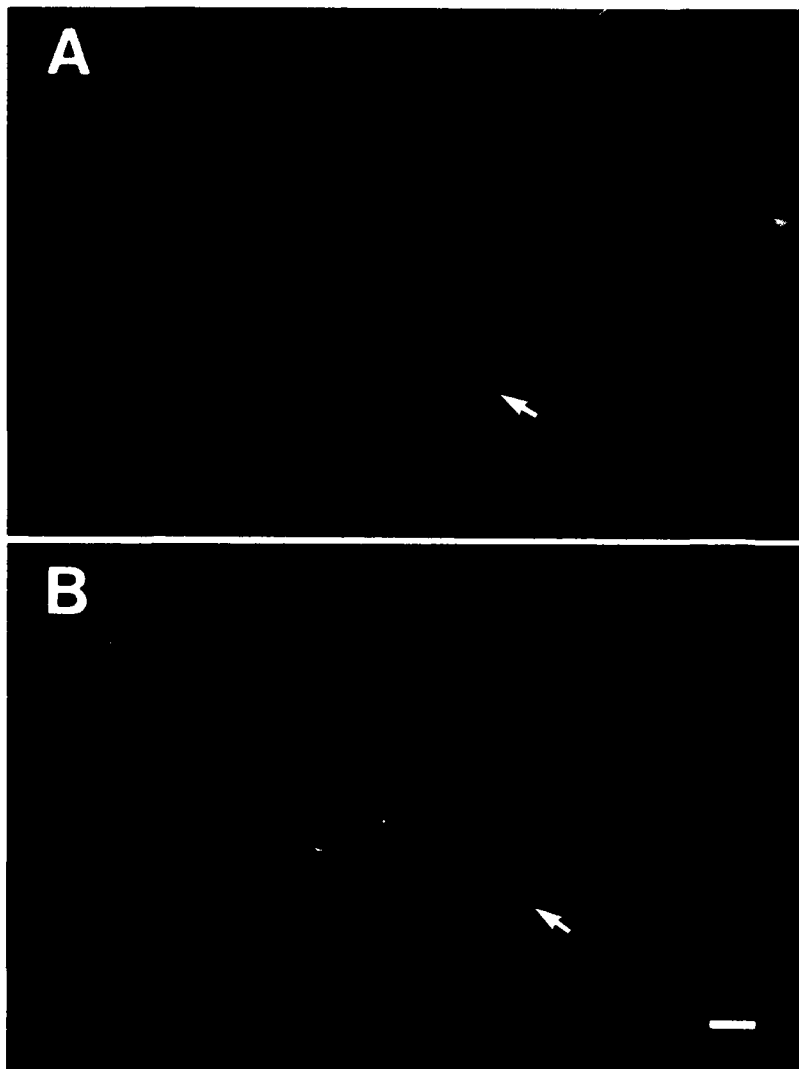


Fig. 7. Double immunofluorescence microscopy of human neutrophils using antibodies against kinesin and tubulin. Fixed neutrophils were incubated with rat monoclonal antitubulin antibodies and antikinesin tail antibodies to detect microtubules and kinesin in the cells. Antitubulin antibodies were detected with rhodamine-conjugated antirat goat antibodies and antikinesin antibodies were detected with fluoresceinated antirabbit goat antibodies (A, tubulin; B, kinesin). Arrows indicate labeling of microtubules in the two panels. Bar, 0.6 μ m.

teins may modulate the ATPase activity of dynein in a manner analogous to the effect of caldesmon on myosin II [23]. It has also been suggested that the kinesin light chain polypeptides that bind to the kinesin heavy chain may regulate kinesin ATPase activity in a manner similar to the regulation of smooth muscle myosin ATPase by myosin light chains [23]. In addition, work by Thaler and Haimo [24] has demonstrated a role for calcineurin phosphatase activity in the regulation of pigment granule movement in the microtubule-based translocation system of fish melanophores.

Neutrophils possess an exocytotic pathway that is highly regulated and therefore may be experimentally useful for defining mechanisms by which stimulus-responsive organelle movement is controlled. In neutrophils, posttranslational modification of granule-associated kinesin may affect the interactions of this protein with accessory regulatory molecules or organelle membranes and may modulate the formation of translocation complexes, representing another form of regulatory control over granule movement.

ACKNOWLEDGMENTS

The authors thank Dr. Jonathan Scholey, University of California, Davis for the *Drosophila* kinesin antibody; Dr. Jeanette C. Bulinski, Columbia University, New York for

tubulin antibodies; and Dr. Douglas B. Murphy for purified bovine brain kinesin used in this study. The authors also gratefully acknowledge the technical assistance of V. Parekh in those studies involving fluorescence microscopy.

REFERENCES

1. Kiehart, D.P. (1990) Molecular genetic dissection of myosin heavy chain function. *Cell* **60**, 347-350.
2. Schliwa, M. (1989) Head and tail. *Cell* **56**, 719-720.
3. Adams, R.J., Pollard, T.D. (1986) Propulsion of organelles isolated from *Acanthamoeba* along actin filaments by myosin-I. *Nature* **322**, 754-756.
4. Kachar, B., Reese, T.S. (1988) The mechanism of cytoplasmic streaming in Characean algal cells: sliding of endoplasmic reticulum along actin filaments. *J. Cell Biol.* **106**, 1545-1552.
5. McIntosh, J.R., Porter, M.E. (1989) Enzymes for microtubule-dependent motility. *J. Biol. Chem.* **264**, 6001-6004.
6. Gibbons, I.R. (1988) Dynein ATPases as microtubule motors. *J. Biol. Chem.* **263**, 15837-15840.
7. Rothwell, S.W., Nath, J., Wright, D.G. (1989) Interactions of cytoplasmic granules with microtubules in human neutrophils. *J. Cell Biol.* **108**, 2313-2326.
8. Yang, J.T., Laymon, R.A., Goldstein, L.B. (1989) A three-domain structure of kinesin heavy chain revealed by DNA sequence and microtubule binding analyses. *Cell* **56**, 879-889.
9. Merrifield, R.B. (1963) Solid phase peptide synthesis. I. The synthesis of a tetrapeptide. *J. Am. Chem. Soc.* **85**, 2149-2154.

10. Wright, D.G., Gallin, J.I. (1979) Secretory responses of human neutrophils: exocytosis of specific (secondary) granules by human neutrophils during adherence in vitro and during exudation in vivo. *J. Immunol.* **123**, 285-294.
11. Taylor, D.L., Condeelis, J.S., Moore, P.L., Allen, R.D. (1973) The contractile basis of amoeboid movement. *J. Cell Biol.* **59**, 378-394.
12. Johnson, C.S., Buster, D., Scholey, J.M. (1990) Light chains of sea urchin kinesin identified by immunoadsorption. *Cell Motil. Cytoskeleton* **16**(3), 204-213.
13. Dentler, W.L., Grannett, S., Rosenbaum, J.L. (1975) Ultrastructural localization of the high molecular weight proteins with in vitro assembled brain microtubules. *J. Cell Biol.* **65**, 237-241.
14. Murphy, D.B., Wallis, K.L. (1983) Isolation of microtubule protein from chicken erythrocytes and determination of the critical concentration for tubulin polymerization in vitro and in vivo. *J. Biol. Chem.* **258**, 8357-8364.
15. Roobol, A., Pogson, C.I., Gull, K. (1980) Identification and characterization of microtubule protein from myxamoebas of *Physarum polycephalum*. *Biochem. J.* **198**, 305-312.
16. Towbin, H., Staehlin, T., Gordon, J. (1979) Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedures and some applications. *Proc. Natl. Acad. Sci. USA* **76**, 4350-4354.
17. Osborne, M., Weber, K. (1982) Immunofluorescence and immunocytochemical procedures with affinity purified antibodies against tubulin-containing structures. *Methods Cell Biol.* **24**, 97-132.
18. Porter, M.E., Scholey, J.M., Stempers, D.C., Vigers, G.P., Vale, R.D., Sheetz, M.P., McIntosh, J.R. (1987) Characterization of the microtubule movement produced by sea urchin egg kinesin. *J. Biol. Chem.* **262**, 2794-2802.
19. Pfister, K.K., Wagner, M.C., Stenien, D.L., Brady, S.T., Bloom, G.S. (1989) Monoclonal antibodies to kinesin heavy and light chain stain vesicle-like structures but not microtubules in cultured cells. *J. Cell Biol.* **108**, 1453-1464.
20. Vallee, R. (1991) Cytoplasmic dynein: advances in microtubule-based motility. *Trends Cell Biol.* **1**, 25-29.
21. Endow, S.A., Hatsumi, M. (1991) A multimember kinesin gene family in *Drosophila*. *Proc. Natl. Acad. Sci. USA* **88**, 4424-4427.
22. Schroer, T.A., Sheetz, M.P. (1991) Two activators of microtubule-based vesicle transport. *J. Cell Biol.* **115**, 1309-1318.
23. Hemric, M.E., Chalovich, J.M. (1990) Characterization of caldesmon binding to myosin. *J. Biol. Chem.* **265**, 19672-19678.
24. Thaler, C.D., Haimo, L.T. (1990) Regulation of organelle transport in melanophores by calcineurin. *J. Cell Biol.* **111**, 1939-1948.

DTIC QUALITY INSPECTED 1

Accession For	
NTIS GRA&I	<input checked="" type="checkbox"/>
DTIC TAB	<input type="checkbox"/>
Unannounced	<input type="checkbox"/>
Justification	
By	
Distribution/	
Availability Codes	
Dist	Avail and/or Special
A-1	20